Title: A novel protein molecule useful for anthrax toxin inhibition in vivo

FIELD OF THE INVENTION:

The present invention relates to a novel molecule useful for anthrax toxin inhibition in vivo. The invention also provides a method for in vivo inhibition of anthrax toxin action using the new molecule.

The main utility of the invention is to develop a candidate molecule for anthrax toxin inhibition and for providing a method for inactivation of toxic activity of a toxin of the nature of anthrax toxin. This molecule has potential for use as therapeutic agent in neutralizing anthrax toxin action in individuals infected with *Bacillus anthracis*

RELATED PRIOR ART:

Anthrax is a bacterial disease caused by *Bacillus anthracis*. The disease primarily affects herbivores but humans can also get infected while dealing with such animals. *B. anthracis* is a potential agent of bioterrorism. Main symptoms comprise dizziness, edema followed by death. The toxic action of anthrax has been attributed to anthrax toxin produced by the bacterium. The toxin can be resolved into three distinct protein components protective antigen (PA), lethal factor (LF) and edema factor (EF). The combination of EF and PA (edema toxin) produces skin edema, while LF and PA (Lethal toxin) are lethal to animals. The three proteins are individually non-toxic. EF is a calcium and calmodulin dependent adenylate cyclase that acts by increasing the intracellular cAMP levels in eukaryotic cells and LF is a Zn^{2+} dependent metalloprotease that leads to increase in IL-1 and TNF-α production by susceptible cells and cleaves several MAP Kinase Kinases (MKK 1, 2 and 3) (Leppla, 1999).

According to the current model of anthrax toxin action, PA binds to an as yet unknown cell surface receptor and gets proteolytically activated by cell surface proteases to PA63. This allows oligomerization and binding of LF/EF. The toxin complex is internalized by receptor mediated endocytosis and is exposed to acidic pH inside the endosome. This change in pH triggers both membrane insertion by PA63 and translocation of LF/EF into the cytosol (Leppla, 1999).

Membrane insertion and channel formation are brought about by a large 2β2-2β3 loop (amino-acid residues 302-325) in the domain II of PA (Petosa et al., 1997). The

loop shows a conserved pattern of alternating hydrophilic and hydrophobic amino-acid residues similar to that observed in *Clostridium perfringens* iota-b toxin. PA has also been shown to possess high degree of homology with the iota-b toxin (Perelle et al., 1993).

Translocation of LF/EF to the cytosol is believed to occur through a channel formed by insertion of heptameric PA63 into the membrane. The formation of ion-conductive channels by PA63 has been demonstrated in both artificial lipid membranes and in CHO-K1 cells. Acidic pH triggers oligomerization, membrane insertion by PA63 and translocation of LF into the cytosol of mammalian cells.

A recombinant vaccine candidate, PA-D, in which furin cleavage site of PA was deleted has been reported by Singh et al., 1989. This recombinant protein (PA-D) was completely non-toxic to macrophage like cell lines as well as when administered in Fischer 344 rats in combination with LF whereas wild-type PA plus LF killed the rats within 60 min. PA-D blocked the action of anthrax toxin albeit at higher concentrations than the wild-type protein due to which this molecule does not seem to be an effective inhibitor of anthrax toxin action. Hence need exists to develop a more potent candidate molecule such as dominant negative inhibitor for anthrax toxin inhibition. No report on dominant negative inhibition of anthrax toxin action exists.

Here we describe for the first time, a novel mutant PA protein which obviates the drawback listed above. It acts as a dominant negative inhibitor of anthrax toxin action. The protein is completely non-toxic both in vitro and in vivo and completely inhibits the lethal effect of the native toxin at equimolar concentrations. This molecule is a better substitute for in vivo inhibition of anthrax toxin in comparison to PA-D since it can inhibit the action of anthrax toxin when present at equimolar or substantially lower concentrations than wild-type protein.

Till date, no such molecule has been reported for inactivation of anthrax toxin action. The approach taken herein for inactivation of anthrax toxin action is a novel one.

OBJECTS OF THE INVENTION:

The main object of the invention is to provide a novel molecule for anthrax toxin inhibition.

Another object is to provide a method for inactivation of toxic activity of a toxin of the nature similar to that of anthrax toxin.

Yet another object of the invention is to provide a therapeutic agent for use in neutralizing anthrax toxin action in individuals infected with *Bacillus anthracis*.

Summary of the invention:

Anthrax is a bacterial disease caused by a gram-positive bacteria *Bacillus anthracis* which affects cattle and humans. Major virulence factor of *B. anthracis* is a tripartite protein exotoxin called anthrax toxin which consists of three proteins: protective antigen (PA), lethal factor (LF) and edema factor (EF).

Present invention provides a candidate molecule, recombinant protective antigen, useful for anthrax toxin inhibition comprising a protein designated as PA-I, wherein the 2β2-2β3 loop containing the residues of the amphipathic loop of the homologous iota-b toxin.

Also is provided DNA sequence of the mutated gene encoding the recombinant protein

The invention also provides a method for construction of the recombinant protein which comprises

PCR based mutagensis of PA gene resulting into dominant negative mutant of PA, purification of mutant PA protein from *B. anthracis*, cytotoxicity assay, in vitro inhibition of pore-forming ability of wild-type PA by PA-I for demonstrating defective channel formation followed by competitive inhibition assay for checking the equivalent activity of the native toxin on mammalian cells and assaying for inhibition of the wild-type toxic activity of anthrax toxin in vivo.

DESCRIPTION OF THE ACCOMPANYING DRAWINGS:

Figure 1: PA and PA-I were purified from the cell supernatants of *B. anthracis* and analyzed on 10% SDS-PAGE. Lane 1: Molecular Weight Marker (kDa) ; Lane 2: Native PA ; Lane 3: PA-I

Figure 2: J774A.1 cells were cultured in 96 well plates in DMEM containing 10% fetal bovine serum and incubated with LF (1 μg/ml) in combination with varying concentrations of PA and PA-I for 3 h at 37 °C. At the end of the experiment, toxicity was determined by MTT assay.

Figure 3: CHO-K1 cells were incubated with PA-I or PA-D mixed with varying concentrations of wild type PA at 37°C for 3 h in combination with $LF^{1-254}.TR.PE^{398-613}$.

At the end of 3 h, cells were incubated with medium containing ^{3}H-leucine (1 μCi/ml) for 1 h at 37 °C. At the end of the experiment, amount of ^{3}H-leucine incorporation was measured. Results are expressed as percentage of ^{3}H-leucine incorporated by viable cells in the absence of added proteins.

Figure 4: CHO-K1 cells, preloaded with $^{86}Rb^{+}$, were incubated with trypsin cleaved PA and PA-I mixed in equimolar ratios at neutral pH for 2 h at 4 °C. After washing twice with cold phosphate buffered saline, the cells were subjected to acidic pH shock. The leakage of $^{86}Rb^{+}$ into the medium was then determined. Results are expressed as percentage of $^{86}Rb^{+}$ associated with cells in the absence of added proteins.

DETAILED DESCRIPTION OF THE INVENTION:

Accordingly, present invention provides a novel molecule, said molecule being a recombinant protective antigen and useful for anthrax toxin inhibition. The molecule comprises a protein designated as PA-I, wherein the 2β2-2β3 loop comprises of the residues of the amphipathic loop of the homologous iota-b toxin are as given below.

Sequence at 2β2-2β3 loop in native PA:

^{302}E V H G N A E V H A S F F D I G G S V S A G F^{324}

'iota b toxin' sequence inserted at 2β2-2β3 loop in the recombinant PA-I:

^{302}V G V S I S A G Y Q N G F T G N I T T S A G F^{324}

The changes in the amino-acid sequence in this loop have rendered it non-toxic and imparted a dominant negative phenotype consequently inhibiting the anthrax toxin action. The mutagenesis of the PA gene in this region has caused inhibition of pore-forming ability of wild-type PA by PA-I by defective channel formation.

The invention also provides the DNA sequence of the mutated gene encoding the recombinant protein comprising:

GTA GGA GTT TCA ATT TCA GCA GGG TAT CAG AAC GGC TTT ACT GGT AAT ATC ACT ACA TCT GCA GGA TTT

Also are provided the steps of the invention comprising:

a. Designing the oligonucleotide primers encompassing the mutation site comprising:

Primer 1:

5'< ATT ACT AAA TCC TGC AGA TGT AGT GAT ATT ACC AGT AAA GCC GTT CTG ATA CCC TGC TGA AAT TGA AAC TCC TAC AGT ATT AGC ATC CCT ACT TGT AGA AGT ATT TTT AC> 3'

Primer 2:

5'< GT GAT TAA TAA AGC TTC TAA TTC > 3'

b. amplifying a portion of the PA gene encoding the mutant region of the 2β2-2β3 loop,

c. cloning the amplified fragment back into the plasmid and inserting the plasmid into *Bacillus anthracis* for expression of the mutated gene,

d. purifying the mutant protein from the culture supernatant of *B. anthracis* followed by characterization of the expressed mutant protein,

e. checking the cytotoxicity of the expressed mutant on mammalian cells in vitro.

f. testing the inhibiting ability of the mutant protein for inhibiting the toxic activity of native PA when present at equimolar or lower concentrations,

g. assaying for the ability of the mutant protein to inhibit pore-forming ability of native PA in vitro,

h. testing the ability of the mutant protein to inhibit anthrax toxin activity in vivo on administration to in-vivo systems in equimolar ratio with wild-type PA plus LF.

In an embodiment the strain used may by *E. coli* or *Bacillus anthracis.*

The vector for cloning the mutant gene may be any expression vector such as plasmid pYS5, pYS6, pMS1, and the like.

The host for expressing the mutant gene can be *E. coli*, Bacillus sp. and the like or a yeast.

In still another embodiment of the invention, mammalian cell lines used can be CHO-K1, J774A.1, RAW 264.7 and the like.

In another embodiment of the invention, concentrations of PA –I used for testing anthrax toxin inhibition can range from 0.01 μg/ml to 0.1 μg/ml.

In yet another embodiment of the invention, in vivo system used to test the in vivo anthrax toxin inhibitory effect can be Fischer 344 rats, guinea pigs, mice and the like.

Other and further aspects, features and advantages of the present invention will be apparent from the following description of the presently preferred embodiments of the invention given for the purpose of disclosures.

EXAMPLE 1:

Reagents:

Biochemicals and reagents were purchased from Sigma Chemical Co., St. Louis, USA. Bacterial culture media was purchased from Difco Laboratories, Becton Dickinson, Delhi, India. The enzymes and chemicals for DNA manipulations were obtained from New England BioLabs, USA. ^{3}H-Leucine were obtained from Amersham Pharmacia Biotech, Piscataway, NJ, USA.

The Chinese Hamster Ovary cell line (CHO-K1) and J774A.1 macrophage cell line were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% calf serum and 50 μg/ml gentamicin sulfate (Life Technologies, Inc., USA) at 37 °C in a CO_2 incubator.

EXAMPLE 2:

Construction of the mutant PA gene:

Mutation in the PA gene was constructed in the plasmid pYS5 (Singh et al., 1989). A non-mutagenic oligonucleotide primer corresponding to nucleotides 2169-2200 and spanning the unique HindIII site was used for PCR with a mutagenic primer corresponding to nucleotides 2785-2860 encompassing the unique PstI site and containing the desired mutations at nucleotides 2792-2851 (nucleotide numbering is according to Welkos et al., 1988). PCR was performed in a 100 ul tube at the following conditions:

94 °C: 1 min.

94 °C: 30 sec

55 °C: 1 min

72 °C: 1 min

72 °C: 10 min

4 °C: 1 h

The constituents of the reaction were:

10X PCR buffer: 1X

Template DNA: 0.5 μg

Forward primer: 0.5 μM

Reverse Primer: 0.5 μM

dNTPs: 20 μM

Taq DNA polymerase: 2.5 U/μl

The amplified PCR product was digested with *Pst*I and *Hind*III as describer below:

10X Buffer: 1X

Template: 10 μg

PstI: 10 U

HindIII: 10 U

and purified on a 1% low melting point agarose gel. The DNA sample was dissolved in 6X sample buffer (final concentration 1X), loaded on low melting point agarose gel and run at 50V. The plasmid pYS5 was digested with the same enzymes, purified on agarose gel and ligated to the mutant fragment. The DNA sequence of the mutant PA gene was verified by DNA sequencing of at least 200 base pairs spanning the mutated region.

EXAMPLE 3:

Expression and purification of recombinant protein PA-I:

The plasmid carrying the desired sequence was transformed into *E. coli dam dcm* strain SCS110. Unmethylated plasmid DNA was purified and used to transform *B. anthracis* BH441. *B. anthracis* was transformed by adding 2 μg of DNA into electrocompetent cells and exposing them to a voltage of 1.5 kV and resistance of 200 Ω. The transformed culture was grown overnight and the cell supernatant was concentrated using concentrator and the protein analyzed using SDS-PAGE.

EXAMPLE 4 :

Molecular weight determination:

The molecular weight of PA-I was determined by SDS-PAGE (Laemlli, 1970). The protein sample (2 μg) was dissolved in 5X SDS dye (final concentration 1X) and run on

the 10 % gel. The molecular weight of PA-I was found to be equal to that of native PA (83 kDa) as determined by SDS-PAGE using appropriate molecular weight standards (Figure 1).

EXAMPLE 5:

Cytotoxicity assay :

To study the cytotoxicity, varying concentrations of PA and PA-I were added to J774A.1 cells together with LF (1.0 μg/ml) and incubated for 3 h at 37°C. At the end of the experiment, cell viability was determined using MTT assay (Singh et al., 1994). The result showed that the mutant PA protein PA-I is completely non-toxic to J774A.1 cells (Figure 2).

EXAMPLE 6:

Inhibition of the activity of native PA by PA-I

Inhibition of activity of native PA by PA-I was investigated by mixing of the mutant PA protein and native type PA at varying ratios resulted in alterations in the cytotoxic activity of the toxin containing the native protein (PA plus LF). When the mutant and native PA were present at equimolar concentrations, complete inhibition in protein synthesis of CHO-K1 cells was observed. A significant inhibition could be detected when the ratio of PA-I to PA was 1:4. These data suggest that the PA-I inhibits native PA mediated cellular intoxication (Figure 3).

EXAMPLE 7:

Inhibition of pore forming ability of native PA by PA I:

Recombinant protein (PA-I) and the native protein (PA) were mixed together (2 μg/ml each) at the neutral pH and incubated with CHO-K1 cells preloaded with $^{86}Rb^{+}$ at 4 °C. After 2 h, the cells were washed to remove unbound proteins and incubated with isotonic buffer of pH 5.0 or 7.0 for 30 min. at 37 °C. Whereas native PA released 62% of the radiolabel from cells, equimolar mixture containing PA and PA-I showed insignificant release of $^{86}Rb^{+}$. The results suggest that there is complete inhibition of channel forming ability of PA by PA-I (Figure 4). The capacity of PA-I to dramatically alter the channel

forming ability of native PA provides evidence that these two species can interact to form dysfunctional hetero-oligomeric structures

EXAMPLE 8:

In vivo inhibition of anthrax toxin activity:

Animal experiments were performed to test the efficacy of PA-I to act as a dominant negative inhibitor of lethal toxin action *in vivo* (that is in equimolar concentration with respect to native PA. Native lethal toxin (40 μg PA + 8 μg LF) resulted in the death of male Fischer 344 rats in approximately 60 min. (Table 1), whereas a 1:1 mix containing native PA and PA-I (40 μg PA + 40 μg PA-I + 8 μg LF) protected rats and no symptoms were evident even after 48 h. Equimolar ratio of native PA and PA-D resulted in the death of rats within 70 minutes.

Table 1. Inhibitory action of PA-I on Fischer 344 rats:

PA (μg)	LF (μg)	PA-I (μg)	PA-D (μg)	TTD [a]
40	-	-	-	Survived
-	8	-	-	Survived
40	40	-	-	60 min.
40	8	-	40	70 min.
40	8	40	-	Survived

[a] TTD is the time to death of Fischer 344 rats after administration of proteins.

Advantages

Main advantage of the invention is in providing a novel recombinant candidate molecule which is a dominant negative inhibitor which inhibitis anthrax toxin action. can be valuable for treatment of anthrax toxin action. Use of *Bacillus anthracis* as a bioweapon has become the bane of the defence establishments in various countries. The recombinant protein also has good potential for use as therapeutic agent for neutralizing anthrax toxin action in individuals infected with *Bacillus anthracis*. The invention also provides a

method for inactivation of toxic activity of a toxin of the nature similar to that of anthrax toxin.

Thus, the present invention provides a novel candidate molecule useful for anthrax toxin inhibition in vivo comprising the recombinant protective antigen (PA) protein. Native PA plus the recombinant protein in equimolar ratio in combination with lethal factor (LF) thereby leading to complete inhibition of native toxin activity. Main utility of the invention is to develop a candidate molecule for anthrax toxin inhibition and for providing a method for inactivation of toxic activity of a toxin of the nature of anthrax toxin. This molecule can be useful as therapeutic agent for use in neutralizing anthrax toxin action in individuals infected with *Bacillus anthracis*.